

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

David H. Gelfand et al.

Application No.: 10/052,417

Filed: January 17, 2002

For: THERMOSTABLE DNA
POLYMERASES INCORPORATING
NUCLEOSIDE TRIPHOSPHATES
LABELED WITH FLUORESCCEIN
DYES

Customer No.: 41504

Confirmation No. 4095

Examiner: Jehanne Souaya Sitton

Technology Center/Art Unit: 1634

DECLARATION UNDER 37 CFR 1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Nancy Jeneane Schoenbrunner, being duly warned that willful false statements and the like are punishable by fine or imprisonment or both (18 U.S.C. § 1001), and may jeopardize the validity of the patent application or any patent issuing thereon, state and declare as follows:

1. All statements herein made of my own knowledge are true, and statements made on information or belief are believed to be true and correct.

2. I received my Ph.D. from Biozentrum der Universität Basel in January, 1997 and an Diplom Biochemie from Universität Bayreuth in December, 1992. I am an employee of Roche Molecular Systems, Inc. ("RMS") and I have been employed by RMS for 10 years. My current position is Research Leader. My work has involved characterization and design of DNA polymerases with novel properties for PCR applications. I develop and carry out

polymerase activity assays, assess substrate preference, enzyme kinetics and perform molecular modeling of the structures of natural and variant polymerases with various substrates. A copy of my CV is attached as Exhibit A.

3. I have reviewed the above-referenced patent application and I understand that the pending claims relate in part to polymerases having a mutation (corresponding to the E681R mutation in *Taq* polymerase, where “R” refers to arginine) that greatly reduces discrimination against fluorescein-labeled nucleotides. I have reviewed the Office Action mailed on April 25, 2008, and I understand that the patent Examiner has rejected the pending claims as obvious in view of any of three Brandis patent publications (US Patent No. 6,256,193; US Patent Publication 2002/0164591; or US Patent Publication 2006/0088879) (referred to jointly here as “Brandis”) in view of Baker (US Patent No. 5,571,706) and Cormier (US Patent No. 5,418,155).

4. According to the April 25, 2008 Office Action, Brandis suggested that substitution with R would improve (reduce) discrimination properties of the mutant polymerase and that the improvement was predictable based on the teachings of Brandis and Baker (Office Action, page 11). The Office Action noted that Brandis provided data of mutations of “all amino acids in the same family as ... R” (Office Action, page 11). Further, the Office Action quoted Baker as stating: “It is well known in the biological arts that conservative amino acid substitutions can be made in protein sequences without affecting the function of the protein” (Office Action, page 3 and again on page 4). The Office Action concluded that:

- (a) an “ordinary artisan would have been motivated to make the additional amino acid mutants... R,” (Office Action page 4)
- (b) it would have been “obvious to try,” (Office Action page 4) and
- (c) the “results would have been predictable” (Office Action page 4).

5. The Office Action indicated that the results presented in the Declaration of Dr. Gelfand (filed 1/3/2003), showing that R resulted in the best reduced discrimination, was not

a surprise because Dr. Gelfand used an assay involving different nucleotides and labels than used in the assay described in Brandis (Office Action, page 11). The Office Action indicated that Brandis predicted that the "degree of discrimination" would vary depending on the "base, dye, or linker" used (Office Action, page 11). The Office Action further stated that the Gelfand results were not a surprise because the level of discrimination for R "was on the same order of magnitude as a number of the same mutants which Brandis teaches" (Office Action, page 12).

6. I disagree with the logic and conclusions of the Office Action. Figure 1 of Dr. Gelfand's Declaration is the only data on the record that shows the effect of all possible 19 amino acid substitutions at the position of interest. Figure 1 of Dr. Gelfand's Declaration is therefore the best source of information on the record to directly compare the effect of different amino acid substitutions. This Figure shows the E683R mutation (corresponding to the E681 position in *Taq* polymerase) provided the best reduced discrimination for a fluorescein (HEX)-labeled dCTP. Specifically, the ratio of amount of fluorescein-labeled dCTP to unlabeled-dCTP required to reduce radioactively-labeled dCMP incorporation by 50% was 0.04. Data for E683H, E683K, and E683M are also provided in Figure 1 of Dr. Gelfand's Declaration and are summarized for convenience as follows:

Mutation	Figure 1 ratio	Fold-improvement of R over other substitution
E683R	0.04	
E683M	0.79	19.75
E683K	0.41	10.25
E683H	1.45	36.25
E683	19.05	476.25

Thus, Dr. Gelfand's data shows that R was almost 20 times better than M, and about 10 and 30-fold better than K and H, respectively.

7. In contrast, data presented by Brandis (e.g., in Table 2 of US Patent No. 6,265,193) indicates that M is the best mutation (showing least discrimination), with H and K ranking somewhere in the middle of the 16 different substitutions presented in Brandis. According to the Office Action (citing Baker): "It is well known in the biological arts that conservative amino acid substitutions can be made in protein sequences without affecting the function of the protein" (Office Action, page 3 and again on page 4). If R, H, and K reflected conservative amino acid changes, and one expected similar results for these substitutions, one would have expected that R would result in levels of discrimination similar to those observed for H and K (i.e., showing a 6-7 -fold improvement). If this was the expectation, it is not clear why one would make the R substitution at all since the H and K substitutions were not particularly good compared to a number of other substitutions. For example, M was 6.71 times "better" than H and 7.83 times "better" than K. Thus, one would have selected M and not proceeded with a substitution expected to work only as well as H or K.

8. Further, if R, H, and K reflected conservative amino acid changes, and one expected similar results for these substitutions, one would not have expected that R would show a 10-30-fold improvement relative to K or H (as observed by Gelfand) and one would certainly not have expected that the *ranking* of substitutions would change to such a great degree that R would be nearly 20-fold better than M. Indeed, if one of ordinary skill in the art expected conservative amino acid changes to have approximately the same effect, and then read the Brandis data, one would have been surprised to find that R was far superior than M as a substitution.

9. The Office Action appears to argue that one of ordinary skill in the art would not be surprised to find that different amino acid substitutions have superior discrimination depending on what type of labeled nucleotide was used in the particular assay involved in the determination. Specifically, the Office Action argued that "the ordinary artisan would have expected the exact levels of discrimination to differ based on the base, dye or linker used in the assay" (Office Action, sentence spanning pages 11-12). In support of this statement, the Office Action cited Brandis (col. 6, line 27-37) as stating that, "The precise degree of

discrimination will also vary in accordance with the specific fluorescently labeled nucleotide assayed, e.g., variations in base, dye, or linker. Mutant DNA polymerase of the invention may exhibit anywhere from a slight reduction in discrimination ... to complete elimination of discrimination." The Office Action argued that "the type of nucleotide (dCTP vs ddCTP) as well as the label (Tet(II) vs HEX-2-PA used in Brandis and Dr. Gelfand's declaration are different" (Office Action, page 11).

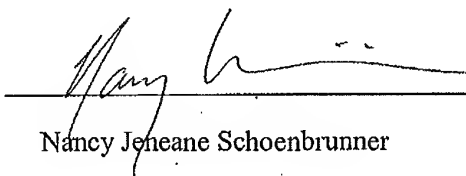
10. While the exact nucleotide and label are different between the Brandis and Gelfand assays, they are not *very* different. As noted in the Office Action, Brandis uses ddCTP, whereas Gelfand uses dCTP. The additional 3' deoxy in ddCTP affects further incorporation of nucleotides to the ddCTP but the portions of a polymerase that interact with the 3' part of a labeled nucleotide are different from those that interact with the label part of a fluorescein-labeled nucleotide. The 3' deoxy position is on the sugar of the nucleotide and very far away from the dye label which is attached to the base. Very different amino acids in the polymerase interrogate the base and moieties attached to the base compared to amino acids that interrogate the sugar. I am not aware of any evidence to date that indicates position 681 in *Taq* polymerase mediates discrimination against 3' deoxy nucleotides. Indeed, discrimination against 3' deoxy nucleotides is mediated by the nature of the amino acid at position 667 in *Taq* DNA polymerase near the active site of the enzyme, not at position 681. (Tabor, S., and Richardson, C. C. (1995) A single residue in DNA polymerases of the *Escherichia coli* DNA polymerase I family is critical for distinguishing between deoxy- and dideoxyribonucleotides, *Proc Natl Acad Sci USA*, 92, 6339-6343). Thus, one of skill in the art would not expect that presence or absence of a 3' hydroxyl (the difference between the nucleotides in Brandis and Gelfand) would affect discrimination against incorporation of fluorescein-labeled nucleotides.

11. The labels used in the Gelfand and Brandis assays are different, but the Tet(II) and HEX labels used by Brandis and Gelfand, respectively, are relatively similar (see attached Figure 1). Both dyes are members of the fluorescein family, the HEX label being substituted with six chloro moieties whereas the TET label has four chloro substitutions. While one would not expect to be able to directly compare quantities between two different assays measuring discrimination, one would expect that the *ranking* of amino acid substitutions would

be similar when the assays use similar labeled nucleotides as is the case here. For example, if one assay showed that M was best and that H and K provided middle-of-the-pack results, one of ordinary skill in the art would generally expect a second assay for measuring similarly-labeled nucleotide discrimination to yield essentially the same ranking of substitutions, i.e., M better than H or K. In view of the similarity between the HEX and TET labels, I would not have expected the relative discrimination between HEX and TET-labelled nucleotides to be significantly different between different amino acid substitutions at position 681. Therefore, it is my opinion that one of ordinary skill in the art would have been surprised to learn that substitution with R results in such a superior level of reduced discrimination compared to other basic amino acid substitutions such as H and K and results in the best substitution overall, having a nearly 20-fold improvement over M.

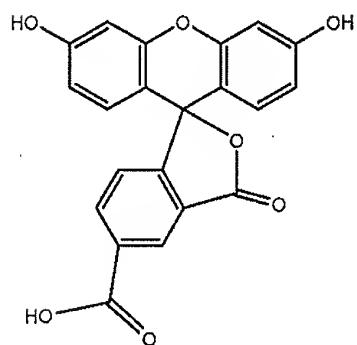
12. In view of the above arguments, I do not believe that the reduced discrimination observed by Gelfand for the R substitution was predictable or expected. If one of skill would have expected similar activity from conservative amino acid changes, one would have expected an R substitution to generate "middle-of-the pack" results similar to the H or K substitutions, not activities that were nearly 10-fold better than the next-best substitution and nearly 20-fold better than M, which Brandis identified as best. Accordingly, it is my opinion that the effect of the R substitution on labeled nucleotide discrimination was not expected or obvious.

Dated: 1/20/2009

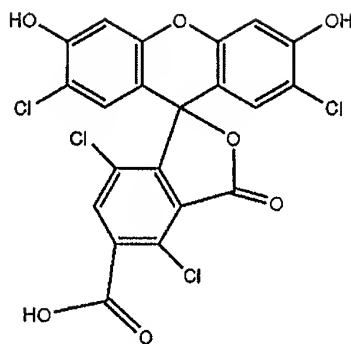


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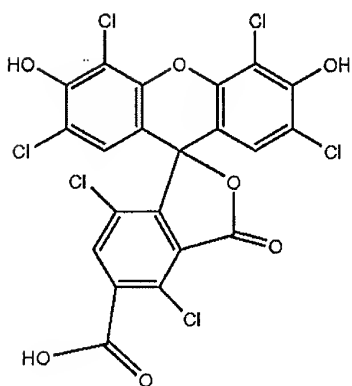
Figure 1



FAM



TET



HEX

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SUMMARY: STRUCTURE-BASED DESIGN OF THERMOSTABLE DNA POLYMERASES WITH NOVEL PROPERTIES; DEVELOPMENT OF ASSAYS FOR THE CHARACTERIZATION OF DESIGNER DNA POLYMERASES AND OTHER DNA ENZYMES; PCR APPLICATIONS OF DESIGNER DNA POLYMERASES AND NUCLEIC ACID MODIFICATIONS; ASSIST OTHERS IN THE COMPANY (PUBLIC RELATIONS, TRAINING) BY GENERATING GRAPHICAL IMAGES FOR VARIOUS PURPOSES. DURING THESE PROJECTS I DIRECTED A SENIOR SCIENTIST TO CARRY OUT THE CLONING, EXPRESSION, PURIFICATION AND CHARACTERIZATION OF DESIGNED ENZYMES.

E x p e r t i s e

- Nucleic Acid Thermodynamic Stability and Structure Prediction Software
- Computer-Aided Molecular Design of Proteins
- Bioinformatics and sequence analysis (GCG package, XSAE, and other Various Databases and Software)
- Programs for Molecular Graphics, Modeling and Dynamics (Moloc, WebLab Viewer, Xplor, Insight II)
- Measurement & computer analysis of complex protein folding and enzyme kinetics
- Development of Enzyme Assays
- Spectroscopic techniques (CD, Fluorescence, UV/VIS)
- Stopped-flow and quench flow techniques
- Limited expertise in NMR
- Fragmentation Analysis of Nucleic Acids using ABI 373, 377 and 3100
- Folding, Stabilization and Chemical Modification of recombinant proteins by various methods
- Programming and operation of Liquid Handling Stations

E d u c a t i o n a l B a c k g r o u n d

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Patents

N. SCHÖNBRUNNER, T. MYERS AND D. GELFAND. THERMOSTABLE OR THERMOACTIVE DNA POLYMERASE MOLECULES WITH ATTENUATED 3'-5' EXONUCLEASE ACTIVITY. U.S. SERIAL No. 10/401,403, FILED ON APRIL 3, 2002.

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Participation in Scientific Meetings

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ANNUAL MEETING OF THE AMERICAN SOCIETY FOR BIOCHEMISTRY AND MOLECULAR BIOLOGY, APRIL 12-15, 2003 SAN DIEGO, CA WITH POSTER PRESENTATION.

GORDON RESEARCH CONFERENCE ON NUCLEIC ACIDS, BRISTOL, RI, JUNE 2-7, 2002 WITH POSTER PRESENTATION.

KEYSTONE SYMPOSIUM ON MOLECULAR MECHANISMS IN DNA REPLICATION AND RECOMBINATION, TAOS, NEW MEXICO 2/16-2/22/1999 WITH POSTER PRESENTATION.

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RESISTANCE, FEB. 25-27, 2000.

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*DEVELOPMENT OF A MICROARRAY BASED HIV-1 DRUG
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UPON REQUEST